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The genetic composition of year class recruits of Cancer magister

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THE GENETIC COMPOSITION OF YEAR CLASS RECRUITS OF *CANCER MAGISTER*

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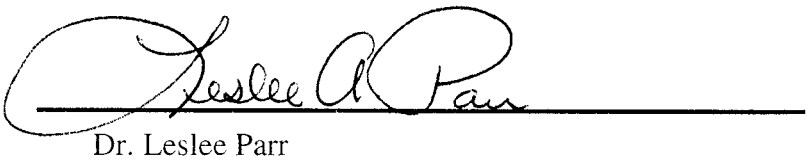
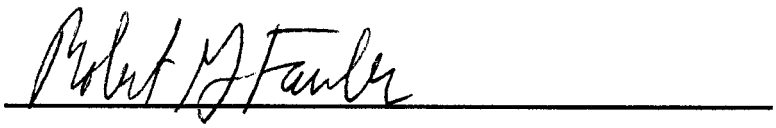


Dr. Leslee Parr



Dr. Robert Fowler

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ABSTRACT

THE GENETIC COMPOSITION OF YEAR CLASS RECRUITS OF *CANCER MAGISTER*

By Sonali Upadhye

Analysis was performed to assess the population structure of an ecologically and commercially important invertebrate species, the Dungeness crab. Genetic variation was examined in the Cytochrome Oxidase I gene of mitochondrial DNA (mtDNA). Wright's inbreeding coefficient (F_{ST}) and Nei's coefficient of gene variation (G_{ST}) were used to determine genetic differentiation among cohorts of megalopae collected on different days. There was no difference (Fst= 0.00) between cohorts recruiting to Coos Bay in late July and early August, whereas the highest between cohort variability was observed between August 1 and August 14 (Fst= 0.92).

There were 29 haplotypes in 200 individual megalopae collected; ninety percent of the haplotypes were singletons (haplotype present in only single individuals). All the haplotypes differ little (1 to 5 base pairs) from the common haplotypes. Phylogenetic analysis reveals the existence of two distinct maternal lineages within the population of Dungeness crabs in Coos Bay.

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Introduction

The long-term goal of this project is to provide scientific information that can guide marine conservation efforts along the Pacific coast. Obtaining a detailed understanding of the dispersal patterns of pelagic marine larvae is challenging because they are minute travelers on vast seas. With the exception of species with very limited dispersal capabilities (Olsen 1985) most information on larval dispersal comes from indirect estimates based on a variety of sources, including oceanography (Limouzy-Paris et al. 1997), species invasions (Carlton & Scanlon 1985; Hicks & Tunnel 1995), and genetics (Palumbi 2001*a*).

Events in the early life history stages of marine organisms, especially those species with a highly dispersing pelagic larval phase, are often critical to the dynamics of the adult populations and perhaps their genetic structure. However, the inability to obtain genetic information from often-microscopic planktonic larvae has been a long-standing problem in marine biology (Olson et al. 1991). The use of PCR-based techniques opens up a wide range of possibilities for population genetic study of relatedness within or differences among larval cohorts (Foltz & Hu 1996).

Dungeness crabs are an important resource in the United States with a growing commercial, tribal, and recreational harvest. The crabs are also an important element in the food chain, depending on other species for food as well as providing food for others. The recent increase in crab harvest raises concern that a possible future over harvest of the resource could occur.

Dungeness crabs occupy ecological niches in both marine and estuarine waters and are important as both predator and prey. Recent studies on the environmental consequences of dredging in estuaries have established a strong probability that the Dungeness crab populations are likely to be seriously reduced by habitat alteration from dredging unless proper precautions are taken to reduce losses (Fernandez et al. 1993). The loss of vital estuarine habitat could significantly reduce recruitment to the offshore fishery.

Marine Protected Areas (MPAs) are the principal management tools in the conservation of marine species and habitats. The criteria for the selection of protected areas include the capability to support healthy populations and to supply larvae to other sites, both inside and outside reserves. To select the most effective network of protected areas, it is important to understand the paths of migration or dispersal of organisms. Most marine species have at least one free-swimming or drifting stage in their life history that is subject to the influence of ocean current dynamics. Evidence shows that larvae often fail to fully achieve their dispersal potential (Burton & Feldman 1982; Knowlton & Keller 1986; Burton & Lee 1994), and recent studies by Swearer et al. (1999) and Jones et al. (1999) indicate that levels of local larval retention may be high. Furthermore, genetic results that indicate high dispersal over evolutionary time scales may often be consistent with very low dispersal over ecological time (Waples 1998; Palumbi 2001*b*). Understanding the ecological connections provided by dispersing larvae has become a high priority in marine conservation biology.

Distribution of Dungeness crabs along the Pacific coast

Cancer magister inhabits sandy and sand-silt habitats in estuaries and near shore zones from Baja California to the Aleutian Islands (Figure 1). Crabs are not common below a depth of 50m (Pauley & Armstrong 1986).

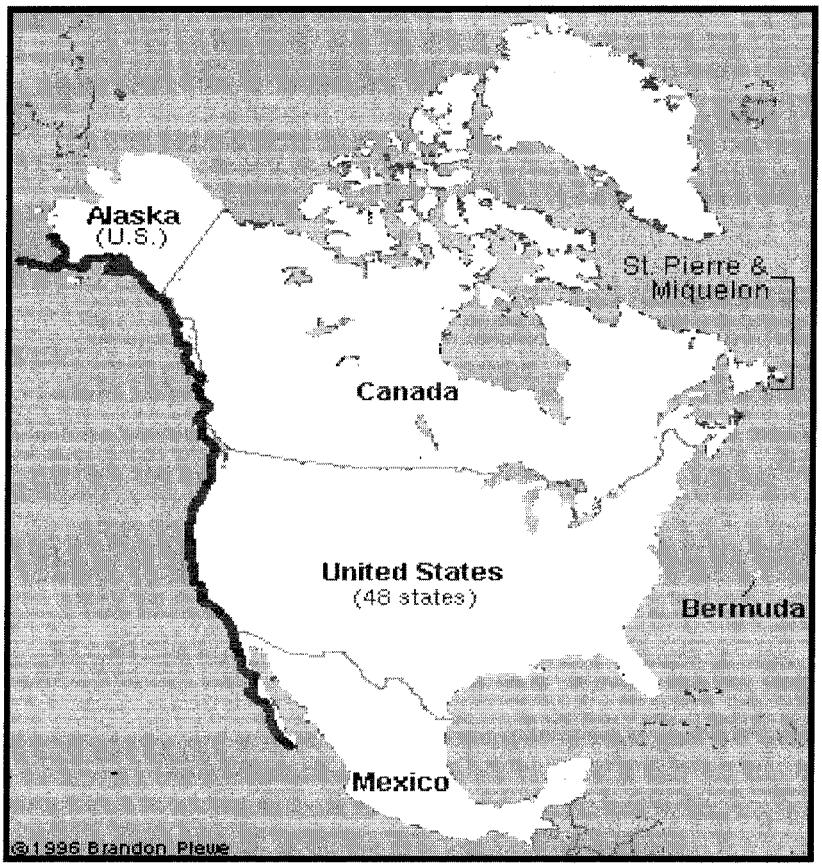

Figure 1. Distribution of Dungeness crabs along the Pacific coast (Cicciomessere 2005).

Life history

The Dungeness crab gets its name from the town of Dungeness, Washington (now called Old Town Dungeness). These crabs live in bays and near shore waters and prefer sandy or muddy bottoms. The range of Dungeness crabs extends from Alaska to south into Mexico (Alaska Department of Fish and Game 1994; Canada's Department of Fisheries and Oceans 2002). They usually live in waters no deeper than 50 meters but can be found at depth of up to 180 meters. The Dungeness crab, like many benthic decapod crustaceans, has a complex life cycle characterized by a relatively long (3 to 4 month) pelagic phase. The timing of breeding and larval development is influenced by latitude. Such a life cycle maximizes the potential for dispersal and mixing of populations. In Washington mating occurs from May to June in offshore locations. Eggs are extruded between October to March in Oregon and October to December in Washington (Canada's Department of Fisheries and Oceans 2002). Males are polygamous, mating with several females per season. Male Dungeness crabs practice mate guarding, and may cradle a female for several days prior to mating. They may also remain with their soft-shelled mates for two days after mating; this offers protection to the female and also ensures that no other males will mate with her. After mating, females store sperm until their eggs are fully developed. Females carry, on average, an estimated 500,000 to 1,000,000 eggs. Fecundity increases with size, and large females may carry up to 2,500,000 eggs. The sperm from one mating may be sufficient to fertilize multiple batches of eggs. Fertilized eggs are brooded under the mother's abdomen for several months until they hatch (Canada's Department of Fisheries and Oceans 2002).

Dungeness crabs begin life as fertilized eggs, which are carried under the female's abdomen until they hatch. Eggs hatch into pelagic larvae that resemble shrimp more closely than adult crabs. Over a period of four to 12 months Dungeness crab larvae undergo a series of six molts before manifesting the characteristic short-tailed form of a juvenile crab (Figure 2). Molting is a normal part of the growth and development process of Dungeness crabs, occurring several times a year for the first two years of the crab's life. Eggs mature in about 2 to 3 months (Cleaver 1949; Wild 1983). The hatching season commonly shortens from north to south along the Pacific coast. The larvae progress through five zoeal stages before molting into megalopae (Reed 1969). The duration of the prezoeal period and the transformation to zoeae varies with salinity. Zoeae first appear within a distance of 5-16 km from shore (Reilly 1983).

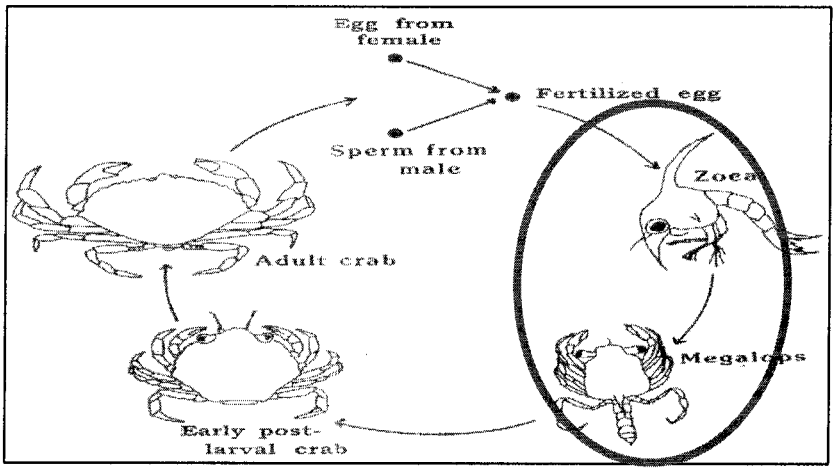

Figure 2. Life cycle of Dungeness crabs (Modified from Reed 1969).

Offshore movement and distribution of larvae probably is regulated by a variety of factors including depth, latitude, temperature, salinity, and ocean currents (Reilly 1983, 1985). Reilly (1983, 1985) used multiple regression to demonstrate that the most important independent variable associated with distribution is depth.

The megalopae is a large (> 10 mm carapace length) and a strong swimmer (up to 40 cm s^{-1}. Its ecological role is to procure a settlement site. For this study, approximately 200 individual megalopae were collected from Coos Bay on four different dates. Advanced megalopae are common in near shore waters (Reilly 1983; Booth et al. 1985) where they settle exclusively in the near shore (< 20 m depth) and estuaries (McConnaughey et al. 1992). Variation in the delivery of megalopae to the coast has long been implicated as a cause of variation in the size of adult populations (Methot 1986; Botsford et al. 1994). The larvae of *Cancer magister* appear to be diurnal vertical migrators. Because horizontal currents vary with depth, it should influence the direction and speed of dispersal. Relatively little is known about the vertical distributions of zoeal stages, especially the late stages that are not sampled (Reilly 1983). Peaks in the concentration of megalopae are commonly reported for samples acquired at night, but megalopae can also be found at the surface during the day, and the number of peaks and timing of occurrence vary amongst studies (Reilly 1983; Booth et al. 1985). Observations indicate megalopae are strongly attracted to light, and, in contrast to most samples collected in the ocean, megalopae within estuarine systems are commonly observed at the surface during the day (Jacoby 1982). Surface accumulations of megalopae generally decline at dawn. Jacoby (1982) speculated that vertical migrations

in response to light and pressure might be important in forming subsurface aggregations of larvae during the day.

Distribution is dependent upon the larval stage, swimming behavior, and ability. Larvae show an overall pattern of vertical distribution. Because horizontal currents vary with depth, the larval behavior influences the direction and speed of their oceanic transport (Reilly 1983, 1985). In oceanic water, megalopae appear to follow a typical diurnal migration pattern with high concentrations at the surface at night, perhaps with peaks near dawn and dusk and low numbers during the day. There is considerable offshore movement of larvae that occurs during the zoeal stages; the larvae appear to be transported seaward from the onset of hatching (Reilly 1983). When megalopae arrive at the lower salinity waters of estuaries they molt into juveniles. Large numbers of juveniles are found in shallow coastal waters and estuaries, living among eelgrass or other aquatic vegetation that provides protection, substrate, and food for these early instars (Stevens & Armstrong 1984, 1985).

The Dungeness crabs attain the sixth or seventh instar by the end of their first year of life (Stevens & Armstrong 1984). The crabs mature after about 2 years at carapace widths of about 116 mm for males and 100 mm for females (Butler 1960). In the third year, molting slows down to approximately once a year. Females must molt their hard shell in order to mate, but males do not have a similar need. Juveniles molt 11 or 12 times prior to sexual maturity. At about 4 years old, most adult Dungeness males in the coastal waters of Washington are of marketable size (> 159 mm) (Cleaver 1949; Williams 1979). Marketable crabs usually molt only once a year (MacKay 1942). The

maximum lifespan of Dungeness crabs is 8 to 10 years. Accurately determining the age of crabs is difficult, as they lose in the molting process many of the characteristics, which are used to define age. Adult Dungeness crabs are found primarily in the ocean but are also abundant in the fresh waters of Washington and British Columbia (Stevens 1982; Stevens et al. 1982). Dungeness crabs migrate offshore during the winter months and return to near shore waters in the spring for breeding (Alaska Department of Fish and Game 1994; Canada's Department of Fisheries and Oceans 2002).

Genetic consequences of dispersal

In general, populations with widely dispersing larvae will be more genetically heterogeneous than populations with restricted dispersal; however, high dispersal potential may not always equate with high genetic homogeneity among populations (Avise 1994). The vertical swimming behavior of larvae and/ or physical retention by ocean currents and eddies has been shown to retard geographic transport of organisms and larvae in both oceanic and estuarine systems (Peterson 1979; Bucklin & Reinecher 1989). For the limited number of species where data are available, there is a strong positive correlation between duration of larval dispersal and the realized dispersal distance (Shanks et al. 2003), and it is generally believed that species with long larval periods disperse widely on ocean currents.

Two ocean currents dominate the oceanographic dynamics of the Pacific coast of North America. The California current system off Washington, Oregon, and northern California consists a southward flowing California current and a northward flowing California undercurrent which surfaces as the Davidson current in the fall and winter

(Hickey 1979). Oceanographic current systems prevalent during the larval dispersal period vary between years, especially between el Niño and la Niña events, and variations of currents can lead to differential transport. The el Niño -southern oscillation (ENSO) phenomenon is an interannual disturbance of the climate system characterized by weak upwelling and warmer surface temperatures in the equatorial Pacific Ocean. ENSO events occur about every 4-7 years. An el Niño year is usually followed by a la Niño, which is dominated by low temperatures and strong upwelling. During el Niño, there is enhanced poleward transport, while during la Nina transport is minimized. The year 2001 was an el Niño year, so the samples were collected during a period of high water transport from the south. Transport of the larvae will depend in part on the predominant currents in this region.

Morphological differentiation of *Cancer magister*

Debrosse et al. (1990) found that *Cancer magister* megalopae differed in number and morphology of spines and setae from Puget Sound and oceanic stocks. They concluded that the differences were probably due to a combination of genotypic and environmental factors unique to distinct spawning stocks, but that there must be some degree of import/export of larvae. Megalopae from the Strait of Georgia are generally smaller and have variations in spine length and setae number compared to their open coast congeners (Debrosse et al. 1990; Dinnel 1993). Stevens et al. (1982) found that carapace width (CW) after the first year averages 44 mm in Grays Harbor, while the range is 63-94 mm in Bodega Bay. If such traits are heritable, it may be possible to detect genetic differences among seasonal cohorts of Dungeness crab megalopae and so

may provide direct evidence of differential dispersal of megalopae along the Pacific coast.

Mitochondrial DNA analysis of the COI gene

Because of the unique matrilineal transmission of mitochondrial DNA, data from mitochondrial DNA sequences are used to draw conclusions about genealogy and evolution. MtDNA is not only useful because of its rapid rate of change or evolution, but also because of its simple, haploid mode of transmission, and because the ease with which substitutions of its base pairs can be compared among taxa (Moritz 1994). Mitochondrial genes have been studied increasingly because of the ease of recovering genetic information that may be useful for investigating molecular and organismal evolution. The predominance of maternal inheritance, lack of extensive recombination, and accelerated rates of nucleotide substitution are features that have favored the use of mtDNA. MtDNA analysis has been used extensively to examine genetic structure in natural populations, and it often shows greater resolution of genetic differences than traditional allozyme polymorphisms (Birky et al. 1989).

Selection of an appropriate gene is a critical strategic and practical decision, with significant consequences for the overall success of the project. Our selection of COI as a target gene to determine genetic variation in Dungeness crab population is supported by published and ongoing work on invertebrates such as Dungeness crabs, Tanner crabs, and Mole crabs (Hebert et al. 2003 & Bucklin et al. 2003).

Project Description

Long-term project objective

There are three long-term objectives for this project. The first is to measure the genetic variability both within and among adult crab populations. Analysis of genetic variation will allow a determination of the degree of gene flow, and hence dispersal, between geographic areas. The assessment of genetic variation is widely used in preserving threatened organisms. The conservation of genetic variation is recognized as an important conservation measure.

The second objective is to measure the genetic variability of groups of returning megalopae within and among geographic sites. Gene flow, the end product of dispersal, can be measured by determining the genetic variability between cohorts of megalopae returning to different geographic sites.

The third long-term objective is to determine the individual origin of each megalopae; a detailed understanding of dispersal trajectories and the magnitude of larval transport are fundamental to understanding the complexities and fate of larvae, including the sources and rates of mortality and the origin of recruits.

Specific objective of current project

The objective of the current project is to meet the second long-term goal by determining the genetic variability within and between groups of returning megalopae during one recruitment season at one location (Coos Bay). Within cohort relatedness will provide an indication of the extent of mixing during the pelagic phase.

Materials and Methods

Sample collection

The 2001 samples were collected from Coos Bay (Figure 3), Oregon, between 43.3 and 44.0 degree N latitude. The heterogeneity of groups of larvae was analyzed during the late settlement period, using mtDNA analysis of the COI gene.

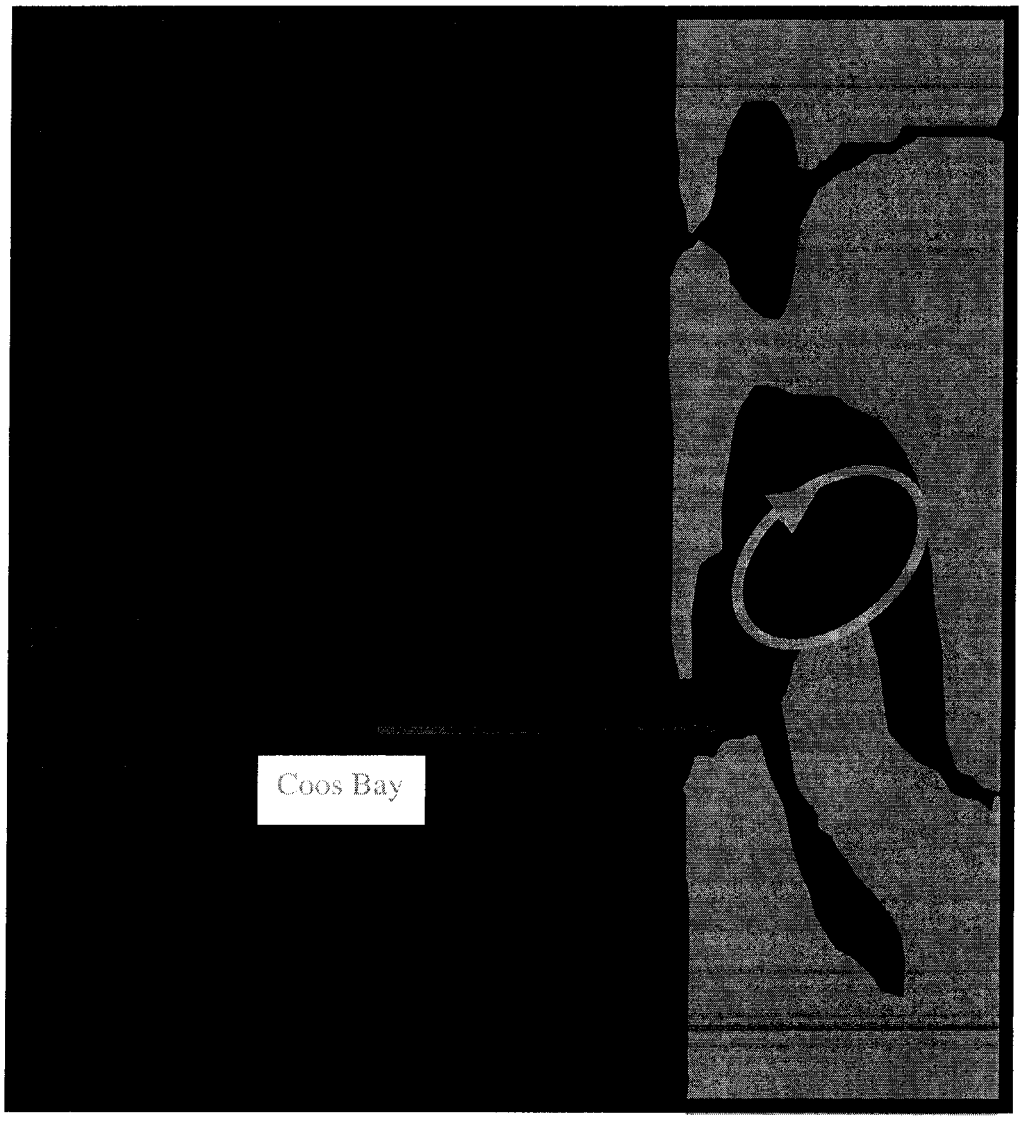

Figure 3. Map of sample collection site, Coos Bay.

Sample collection method

Returning (recruiting) cohorts of final stage megalopae were collected on 4 days (July 26, August 1, August 14, and September 9) during the peak of the recruitment season. Light traps function as behavioral samplers because they depend upon the positive phototropism of megalopae (Figure 4). The traps, which floated at the surface, were constructed from semiopaque 5 gallon plastic jugs. Plankton gained entry through 1x 3 cm openings cut into the apex of translucent plastic funnels. The light source was a 6 Watt AC fluorescent bulb sealed in an acrylic tube. Captured specimens were concentrated in a cod end (250 micron mesh) attached to the base of the trap and sorted in the laboratory into individual containers. Light traps were located at two locations in Coos Bay, one near the Distant Fleet Facility station and the other near the Outer Boat Basin. Although light traps were set for ten days from July until the September 2001 recruitment period, megalopae appeared in the trap only on the 4 days mentioned. This makes sense in light of previous studies, which had determined that larvae occur in the light traps in distinct pulses of abundance (Shanks & Roegner 2001).

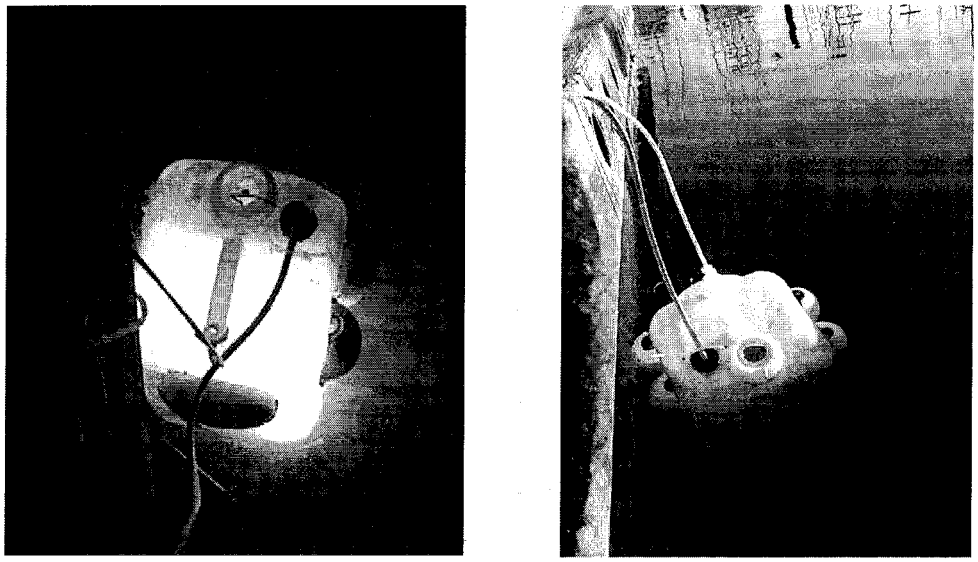

Figure 4. Collection in estuaries with behavioral light traps (picture used with permission from Dr. Leslee Parr).

DNA extraction

Whole body samples of megalopae were used for extraction. DNA was extracted using the Wizard Genomic Purification Kit (Promega). Total genomic DNA was extracted from crab tissue by using the standard SDS/proteinase K digestion (Sambrook 1989). Cell lysis (100ml), nuclei lysis (50ml), protein precipitation (25ml), DNA rehydration (50ml), and RNase (250μl) solutions were used for purifying the extracted DNA. Extracted DNA was labeled and stored at –20 degree C (Table 1) until needed.

PCR amplification

Amplification was preformed in an Eppendorf Mastercycler thermocycler (model number 5332). Reaction volumes totaled 25 µl. These primers specifically amplify 450 bps of the mitochondrial Cytochrome Oxidase I (COI) gene. A standard PCR reaction included 1 µl DNA, 0.5 µM concentration of forward and reverse primers, 1X PCR buffer (10X buffer with 1.5mM Mg was supplied with MasterTaq Kit), 5mM MgCl2, 0.16 mM dNTPs, and 1.0 U Taq DNA polymerase. The following conditions were used for amplification: 96°C for 5 minutes, 25 cycles of 96°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. If double banding occurred, annealing temperature, primer concentration, and magnesium ion concentration were varied as needed to optimize amplification conditions.

Primer sequence

Primers for PCR were ordered from Operon. Genetic variability in the larvae of *Cancer magister* was determined using the sequence analysis of the mitochondrial COI gene. Primers were designed based on a sequence published by Harrison & Crespi (1999). These primers are specific to the mitochondrial COI gene of Dungeness crab. The primer sequences are as follows:

Mitochondrial reverse primer (5'-GTACAGGGAGGGATAGTAGT-3')

Mitochondrial forward primer (5'-GGAGGATTTGGAAATTGATT-3')

Agarose gel electrophoresis

Agarose gels (2%) were made and run with a 100 bp marker (BioLabs) to see if there was successful PCR amplification of extracted DNA. After electrophoresis, the

gels were stained with ethidium bromide for 30 minutes, and then the electrophoretic patterns of the gels were recorded using the Gel Doc 1000 Image Analyzer (BioRad).

PCR cycle sequencing

Cycle sequencing was performed in the Eppendorf Mastercycler thermocycler, using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit, with Ampli Taq Polymerase (Applied Biosystems). Reactions totaling a volume of 20 µl were made. In a standard reaction, 8.0 µl of Terminator Ready Reaction Mix (Applied Biosystems), 1 µl of forward primer, and 4 µl PCR product was added to 0.5 ml thin walled PCR tubes. The following conditions were used for amplification: 30 cycles of rapid thermal ramp at 96°C, 96°C for 10 seconds, rapid thermal ramp to 50°C, 50°C for 5 seconds, rapid thermal ramp to 60°C, 60°C for 4 minutes, and finally, rapid thermal ramp to 4°C (Applied Biosystems). Contents in each PCR tube were spun down to prepare for purification and sequencing of the extension products.

Purification and sequencing of extension products

PCR product was sequenced using the ABI prism Dye Terminator Cycle sequencing Ready Reaction Kit. Plasmid (pGEM3Zf) and water were used as positive and negative controls, respectively. Contents of each extension (PCR) reaction were placed into microcentrifuge tubes containing a sodium acetate/ethanol mixture, and mixed thoroughly. Each tube was then vortexed and placed on ice for 10 minutes to precipitate the extension products. Next, the tubes were spun down for 15-30 minutes and then the supernatant was removed. The remaining pellet was then rinsed with 70%

ethanol, microcentrifuged, and again the supernatant was removed. The pellet was dried before sequencing using the ABI Prism (model 310) sequencer.

Data analysis

Sequences obtained from 200 samples were aligned using the computer program ClustalW in Bioedit (Pearson & Lipman 1988). Estimates of within and between population genetic variations were obtained for all of the samples in the form of haplotype and nucleotide diversities by using DnaSP program version 3.0 (Rozas & Rozas 1999). Haplotype diversity is defined (Nei 1987, equations 8.4 and 8.12) as the probability that two haplotypes randomly chosen from a population are different.

Nucleotide diversity, Pi (p), is the average number of nucleotide differences per site between two sequences (Nei 1987, equations 10.5 or 10.6; see also Nei & Miller 1990) and its sampling variance (Nei 1987, equation 10.7). Nucleotide diversity using Jukes & Cantor correction (1969) is the average number of nucleotide substitutions per site between two sequences (Lynch & Crease 1990, equations 1-2). The Jukes & Cantor (1969) correction was performed in each pairwise comparison; the Pi (p) estimates were obtained as the average of the values for all comparisons.

Nonrandom association between nucleotide variants at different polymorphic sites was calculated using command linkage disequilibrium (Rozas & Rozas 1999). Sites containing alignment gaps, or polymorphic sites segregating for three or four nucleotides, were completely excluded from the analysis. The analysis was performed with all polymorphic sites in the data. The distribution of haplotypes between population samples

was evaluated for homogeneity using the Chi-square test and the Fisher's exact test using the DnaSP program (Snedecor & Cochran 1989).

Differences among populations were quantified by the use of one of several statistics, including Wright's inbreeding coefficient (F_{ST}) (Wright 1978) and Nei's coefficient of gene variation (G_{ST}) (Nei 1975) using the DnaSP program. These coefficients express how heterozygosity is partitioned within and among populations, based on differences in allele frequencies (Wright 1969; Nei 1975; Chai 1976; Wright 1978). Values of F_{ST} and G_{ST} vary from 0 to 1 (Nei 1975; Hedrick 1985; Crow 1986). G_{ST} in particular has a number of useful properties: it can be used for one or many loci, mutation rates do not alter the statistic significantly, the exact number of populations need not be specified, and the statistic is relatively responsive to changes in allele frequencies in time. Although they have important conceptual differences, in practice F_{ST} and G_{ST} are used in a similar fashion as indices of genetic difference among populations (Crow 1986).

Construction of phylogenetic tree

Phylogenetic relationships among the nucleotide sequences were inferred using neighbor joining and minimum evolution network. A phylogenetic tree is a graph composed of nodes and branches, in which only one branch connects any two adjacent nodes. The nodes represent taxonomic units and the branches define the relationships among the units in terms of descent and ancestry (Li 1997). The trees give an indication of the degree of dissimilarity among the populations; the smaller the branches separating

a pair of populations, the more genetically similar are individuals sampled from those patches or year.

The Tamura-Nei model was used to calculate pairwise sequence divergences and then the resulting distances were clustered using minimum evolution, neighbor joining, and interior branch test of phylogeny. A bootstrap test with 500 replications was run to test the confidence of the topology of the phylogenetic tree by using MEGA software, version 3.0 (Kumar et al. 1994). Bootstrap values were calculated for the phylogenetic trees.

Results

A total of 29 haplotypes were seen in the 200 individual megalopae analyzed in this study (Table 1). All samples were collected at two sites in Coos Bay on four days during the recruitment season.

Table 1. Sample identification (samples collected in 2001).

Serial number	Sequence name	Haplotype number	Collection Date
1	010726CB11	H 1	July 26, 2001
2	010726CB12	H 1	July 26, 2001
3	010726CB13	H 1	July 26, 2001
4	010726CB14	H 1	July 26, 2001
5	010726CB15	H 1	July 26, 2001
6	010726CB16	H 1	July 26, 2001
7	010726CB17	H 1	July 26, 2001
8	010726CB18	H 1	July 26, 2001
9	010726CB19	H 1	July 26, 2001
10	010726CB20	H 1	July 26, 2001
11	010726CB21	H 1	July 26, 2001
12	010726CB22	H 1	July 26, 2001
13	010726CB23	H 1	July 26, 2001
14	010726CB24	H 1	July 26, 2001
15	010726CB25	H 1	July 26, 2001
16	010726CB26	H 1	July 26, 2001
17	010726CB27	H 1	July 26, 2001
18	010726CB28	H 1	July 26, 2001
19	010726CB29	H 1	July 26, 2001
20	010726CB30	H 1	July 26, 2001
21	010726CB31	H 1	July 26, 2001
22	010726CB32	H 1	July 26, 2001
23	010726CB33	H 1	July 26, 2001
24	010726CB34	H 1	July 26, 2001
25	010726CB35	H 1	July 26, 2001
26	010726CB36	H 1	July 26, 2001
27	010726CB37	H 1	July 26, 2001
28	010726CB38	H 1	July 26, 2001
29	010726CB39	H 1	July 26, 2001
30	010726CB40	H 1	July 26, 2001
31	010726CB41	H 1	July 26, 2001
32	010726CB42	H 1	July 26, 2001
33	010726CB42	H 1	July 26, 2001

34	010726CB43	H 1	July 26, 2001
35	010726CB44	H 1	July 26, 2001
36	010726CB45	H 1	July 26, 2001
37	010726CB46	H 1	July 26, 2001
38	010726CB47	H 1	July 26, 2001
39	010726CB48	H 1	July 26, 2001
40	010726CB49	H 1	July 26, 2001
41	010726CB50	H 1	July 26, 2001
42	010726CB51	H 1	July 26, 2001
43	010726CB52	H 1	July 26, 2001
44	010726CB53	H 1	July 26, 2001
45	010726CB54	H 1	July 26, 2001
46	010726CB55	H 1	July 26, 2001
47	010726CB56	H 1	July 26, 2001
48	010726CB57	H 1	July 26, 2001
49	010726CB58	H 1	July 26, 2001
50	010801CB36	H 1	August 1, 2001
51	010801CB37	H 1	August 1, 2001
52	010801CB38	H 1	August 1, 2001
53	010801CB39	H 1	August 1, 2001
54	010801CB40	H 1	August 1, 2001
55	010801CB41	H 1	August 1, 2001
56	010801CB44	H 1	August 1, 2001
57	010801CB46	H 1	August 1, 2001
58	010801CB47	H 1	August 1, 2001
59	010801CB48	H 1	August 1, 2001
60	010801CB53	H 1	August 1, 2001
61	010801CB55	H 1	August 1, 2001
62	010801CB56	H 1	August 1, 2001
63	010801CB57	H 1	August 1, 2001
64	010801CB58	H 1	August 1, 2001
65	010801CB59	H 1	August 1, 2001
66	010801CB62	H 1	August 1, 2001
67	010801CB64	H 1	August 1, 2001
68	010801CB84	H 1	August 1, 2001
69	010801CB83	H 1	August 1, 2001
70	010801CB82	H 1	August 1, 2001
71	010801CB81	H 1	August 1, 2001
72	010801CB69	H 1	August 1, 2001
73	010801CB70	H 1	August 1, 2001
74	010801CB71	H 1	August 1, 2001
75	010801CB72	H 1	August 1, 2001
76	010801CB73	H 1	August 1, 2001
77	010801CB74	H 1	August 1, 2001
78	010801CB75	H 1	August 1, 2001

79	010801CB76	H 1	August 1, 2001
80	010814CB06	H 1	August 14, 2001
81	010814CB07	H 1	August 14, 2001
82	010814CB08	H 1	August 14, 2001
83	010814CB10	H 1	August 14, 2001
84	010814CB11	H 1	August 14, 2001
85	010814CB12	H 1	August 14, 2001
86	010814CB13	H 1	August 14, 2001
87	010814CB14	H 1	August 14, 2001
88	010814CB15	H 1	August 14, 2001
89	010814CB16	H 2	August 14, 2001
90	010814CB17	H 2	August 14, 2001
91	010814CB18	H 2	August 14, 2001
92	010814CB19	H 2	August 14, 2001
93	010814CB20	H 2	August 14, 2001
94	010814CB21	H 2	August 14, 2001
95	010814CB22	H 2	August 14, 2001
96	010814CB23	H 2	August 14, 2001
97	010814CB24	H 2	August 14, 2001
98	010814CB25	H 2	August 14, 2001
99	010814CB26	H 2	August 14, 2001
100	010814CB27	H 2	August 14, 2001
101	010814CB28	H 2	August 14, 2001
102	010814CB29	H 2	August 14, 2001
103	010814CB30	H 2	August 14, 2001
104	010814CB31	H 2	August 14, 2001
105	010814CB32	H 2	August 14, 2001
106	010814CB33	H 2	August 14, 2001
107	010814CB34	H 2	August 14, 2001
108	010814CB35	H 2	August 14, 2001
109	010814CB36	H 2	August 14, 2001
110	010814CB37	H 2	August 14, 2001
111	010814CB38	H 2	August 14, 2001
112	010814CB39	H 2	August 14, 2001
113	010814CB40	H 2	August 14, 2001
114	010814CB41	H 2	August 14, 2001
115	010814CB42	H 2	August 14, 2001
116	010814CB43	H 2	August 14, 2001
117	010814CB44	H 2	August 14, 2001
118	010814CB45	H 2	August 14, 2001
119	010814CB46	H 2	August 14, 2001
120	010814CB47	H 2	August 14, 2001
121	010814CB48	H 2	August 14, 2001
122	010814CB49	H 2	August 14, 2001
123	010814CB50	H 2	August 14, 2001

124	010814CB51	H 2	August 14, 2001
125	010814CB52	H 2	August 14, 2001
126	010814CB53	H 2	August 14, 2001
127	010814CB54	H 2	August 14, 2001
128	010814CB55	H 2	August 14, 2001
129	010814CB56	H 2	August 14, 2001
130	010909CB10	H 1	September 9, 2001
131	010909CB11	H 1	September 9, 2001
132	010909CB12	H 1	September 9, 2001
133	010909CB13	H 1	September 9, 2001
134	010909CB14	H 1	September 9, 2001
135	010909CB15	H 2	September 9, 2001
136	010909CB18	H 2	September 9, 2001
137	010909CB16	H 2	September 9, 2001
138	010909CB17	H 2	September 9, 2001
139	010909CB20	H 2	September 9, 2001
140	010909CB21	H 2	September 9, 2001
141	010909CB22	H 2	September 9, 2001
142	010909CB24	H 2	September 9, 2001
143	010909CB25	H 2	September 9, 2001
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145	010909CB27	H 2	September 9, 2001
146	010909CB28	H 2	September 9, 2001
147	010909CB29	H 2	September 9, 2001
148	010909CB30	H 2	September 9, 2001
149	010909CB31	H 2	September 9, 2001
150	010909CB32	H 2	September 9, 2001
151	010909CB33	H 2	September 9, 2001
152	010909CB34	H 2	September 9, 2001
153	010909CB35	H 2	September 9, 2001
154	010909CB36	H 2	September 9, 2001
155	010909CB37	H 2	September 9, 2001
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161	010909CB43	H 2	September 9, 2001
162	010909CB44	H 2	September 9, 2001
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167	010909CB49	H 5	September 9, 2001
168	010909CB50	H 5	September 9, 2001

169	010909CB51	H 6	September 9, 2001
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173	010909CB55	H 9	September 9, 2001
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175	010909CB57	H11	September 9, 2001
176	010909CB58	H11	September 9, 2001
177	010909CB59	H 11	September 9, 2001
178	010909CB60	H 11	September 9, 2001
179	010909CB61	H 11	September 9, 2001
180	010909CB62	H 11	September 9, 2001
181	010909CB63	H 11	September 9, 2001
182	010909CB64	H12	September 9, 2001
183	010909CB65	H 13	September 9, 2001
184	010909CB66	H 14	September 9, 2001
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186	010909CB68	H 16	September 9, 2001
187	010909CB69	H 17	September 9, 2001
188	010909CB70	H 18	September 9, 2001
189	010909CB71	H 19	September 9, 2001
190	010909CB72	H 20	September 9, 2001
191	010909CB73	H 21	September 9, 2001
192	010909CB74	H 22	September 9, 2001
193	010909CB75	H 23	September 9, 2001
194	010909CB76	H 24	September 9, 2001
195	010909CB77	H 24	September 9, 2001
196	010909CB78	H 25	September 9, 2001
197	010909CB79	H 26	September 9, 2001
198	010909CB80	H 27	September 9, 2001
199	010909CB81	H 28	September 9, 2001
200	010909CB82	H 29	September 9, 2001

Sequence diversity

Haplotype frequency distribution for all the 29 haplotypes was calculated. Haplotypes H1 and H2 were present in 80% of the individuals. The rest of the haplotypes, with the exception of H11, were present in one or two individuals (Table 2).

Table 2. Distribution of the 29 haplotypes among the 200 samples.

Haplotype ID	Number of haplotypes	Haplotype frequency %
H1	83	41.50
H2	81	40.50
H3	1	0.50
H4	1	0.50
H5	2	1.00
H6	1	0.50
H7	1	0.50
H8	1	0.50
H9	2	1.00
H10	1	0.50
H11	7	3.50
H12	1	0.50
H13	1	0.50
H14	1	0.50
H15	1	0.50
H16	1	0.50
H17	1	0.50
H18	1	0.50
H19	1	0.50
H20	1	0.50
H21	1	0.50
H22	1	0.50
H23	1	0.50
H24	2	1.00
H25	1	0.50
H26	1	0.50
H27	1	0.50
H28	1	0.50
H29	1	0.50
Total	200	100.00

The haplotype diversity is higher for samples collected during September 9, 2001. Overall the haplotype diversity is high (66.50 %) and nucleotide diversity is low (0.53%). The mean genetic distance within population is low for all samples (Table 3). It is higher for samples collected during September 9, 2001 compared to the rest of the days. For samples collected during July 26, 2001, and August 1, 2001, the nucleotide diversity, haplotype diversity and mean genetic distance within population is 0%, as only one haplotype is present.

Table 3. Measures of genetic diversity in Dungeness crab megalopae collected during 2001.

Groups	Sample size	Nucleotide diversity %	Haplotype diversity %	Mean genetic distance within recruitment cohort
July 26, 2001	49	0	0	0
August 1,2001	30	0	0	0
August 14, 2001	50	.068	15.02	.001
September 9, 2001	71	1.053	75.09	.011
All samples	200	.537	66.50	.005

Fisher's exact test and the Chi-square test

Both the two-tailed Fisher's exact test and the Chi-square test were computed to determine whether the associations between variability at polymorphic sites are, or are not, significant (Sokal & Rohlf 1981). The Bonferroni correction for multiple tests was performed to avoid spurious rejections of the null hypothesis in multiple tests (assuming that all tests are independent) (Weir 1996).

DnaSP calculated the probability associated with a particular chi-square value (with one degree of freedom) using the trapezoidal method of numeric integration (Table 4). Significant disequilibrium was calculated by the Bonferroni procedure for a = 0.05 as indicated by the letter B.

Table 4. Chi-square values and Fisher's exact test shown for significant polymorphic sites.

Site 1	Site 2	Fisher	Chi-square
11	12	0.000***B	200.000***B
11	52	0.107	7.697**
12	52	0.107	7.697**
15	23	0.000***B	200.000***B
15	43	0.020*	48.995***B
23	43	0.020*	48.995***B
43	52	0.107	7.697**
52	76	0.003*	34.711***B
52	164	0.050*	7.429**
54	113	0.107	7.697**
76	164	0.001**	55.700***B
113	191	0.001**	9.718

* 0.01<P<0.05; ** 0.001<P<0.01; *** P<0.001
B, significant by the Bonferroni procedure

The genetic differentiation was calculated among populations, which were collected on different days. In species with low Gst values the majority of variation is

found within population. Samples collected from September 9, 2001 have lower Gst values compared to the rest of the samples (Table 5). Fst, a measure of population divergence, ranges from zero to one. Zero indicates no population divergence, and one indicates population differentiation.

Table 5. Gene flow and genetic differentiation. Selected region: 1-222.

Recruitment cohorts	Number of sequences	Number of segregating sites	Haplotype diversity %	Nucleotide diversity %	Nucleotide diversity with JC
July 26	49	S: 0	0.00	Pi: 0.00000	PiJC: 0.00
August 1	30	S: 0	0.00	Pi: 0.00000	PiJC: 0.00
August 14	50	S: 1	15.02	Pi: .068	PiJC: 0.068
September 9	71	S: 26	75.09	Pi: 1.053	PiJC: 1.065

Fst between 0.0 and 0.10 indicates little population divergence and high gene flow (Wright 1978), while Fst between 0.10 and 0.20 indicates moderate levels of population divergence (a reduction in gene flow allows the accumulation of many allele frequency differences but few if any fixed allelic differences). If the Fst value is greater than 0.20 it indicates high levels of population divergence (population isolation allows the accumulation of fixed allelic differences). The Fst values for September 9, 2001, August 14, 2001, and July 26, 2001, indicate high levels of population divergence. For samples collected during August 01, 2001, the samples showed no population divergence (Table 6).

Table 6. Genetic differentiation among populations.

Recruitment cohort one	Recruitment cohort two	Gst	Fst
July 26	August 1	1.00000	0.00000
July 26	August 14	0.84775	0.91837
July 26	September 9	0.40460	0.28461
August 1	August 14	0.80841	0.91837
August 1	September 9	0.33157	0.28461

Haplotype frequency distribution

On July 26, 2001, and August 1, 2001, only one haplotype (H1) was present in all the individuals. The first finding is that only one haplotype was present in all the individuals on these two days so all these samples share the same ancestor (Figures 5 and 6).

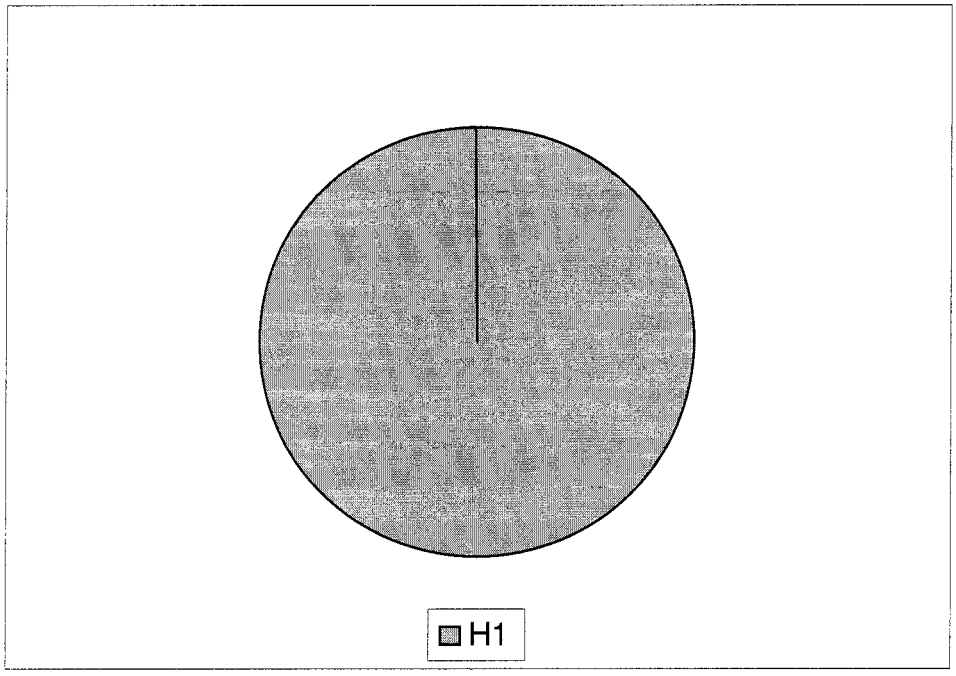

Figure 5. Sequence haplotype frequencies for Dungeness crab megalopae sampled opportunistically at Coos Bay during recruitment season July 26, 2001. H = Haplotype. Sample size = 49.

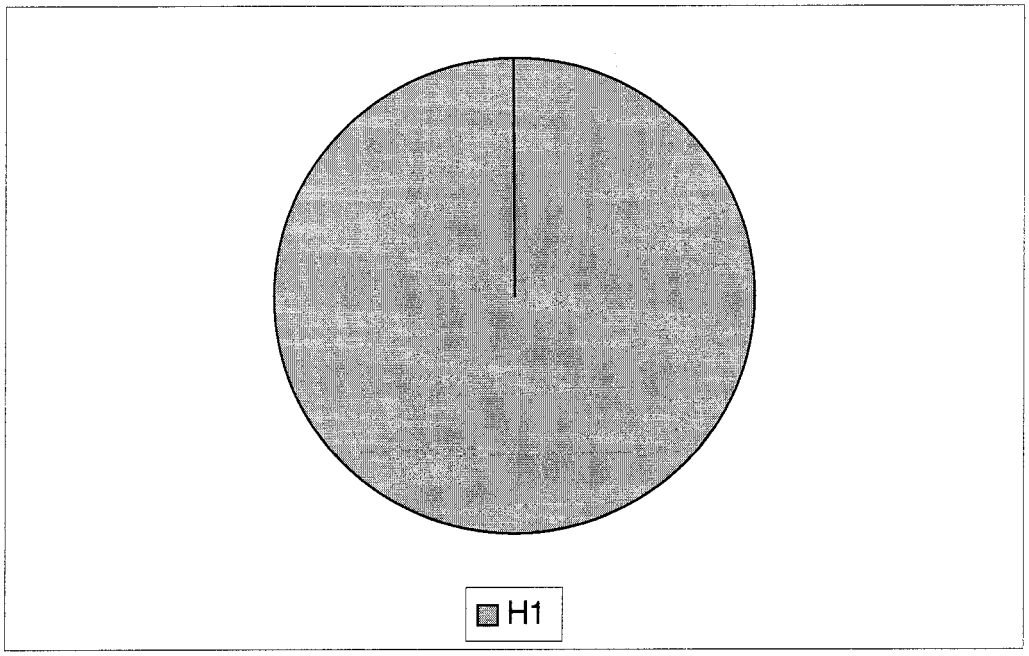

Figure 6. Sequence haplotype frequencies for Dungeness crab megalopae sampled opportunistically at Coos Bay during recruitment season August 01, 2001. H = Haplotype. Sample size = 30.

On August 14, 2001, two haplotypes (H1 and H2) were present (Figure 7). These two haplotypes are the most common haplotypes in all tested populations or, in other words, these are the local haplotypes that have been inherited by a fair proportion of individuals.

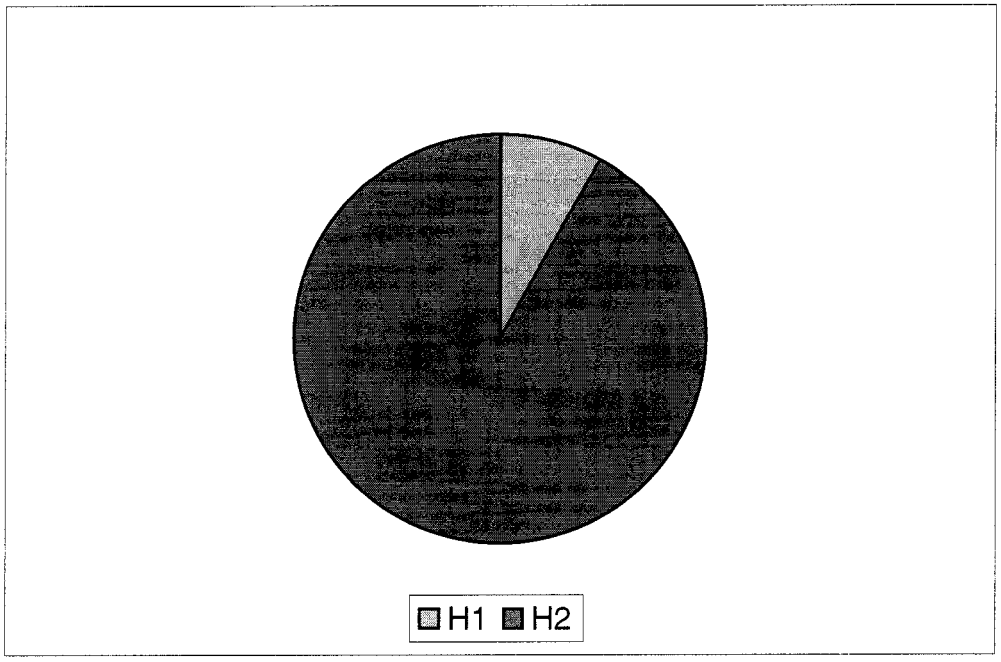

Figure 7. Sequence haplotype frequencies for Dungeness crab megalopae sampled opportunistically at Coos Bay during recruitment season August 14, 2001. H = Haplotype. Sample size = 50.

On September 9, 2001, 29 haplotypes were present. All of the haplotype and nucleotide diversity was due to the 71 samples collected on September 9. This can be due to larvae coming from north and extensive mixing going on during the late settlement period in Coos bay on this day (Figure 8).

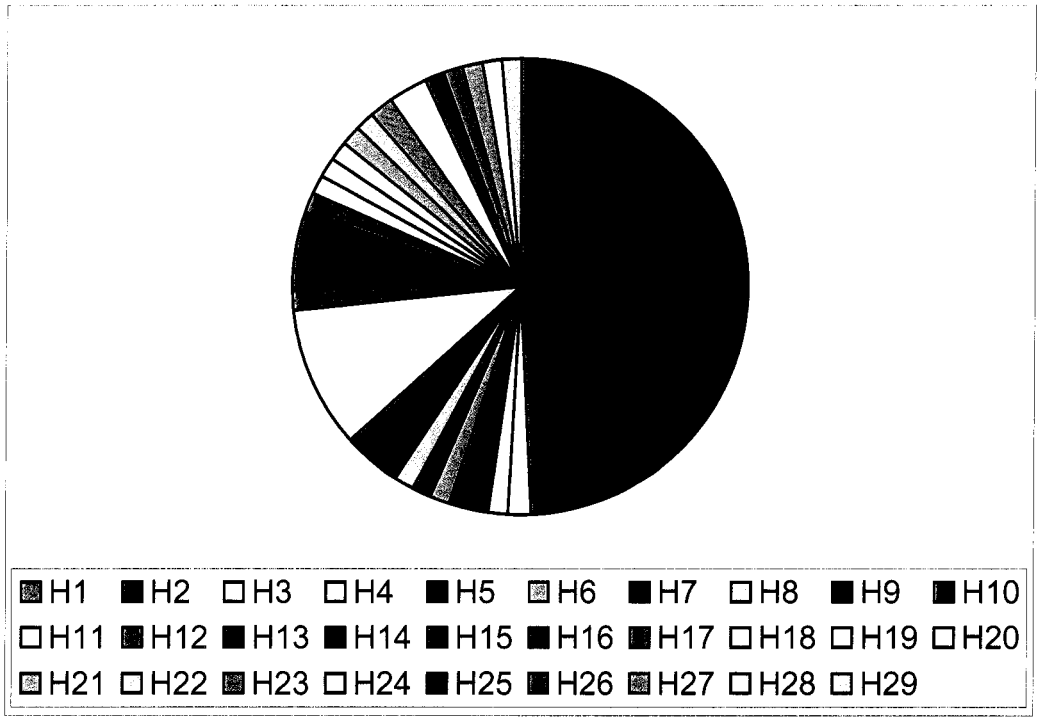

Figure 8. Sequence haplotype frequencies for Dungeness crab megalopae sampled opportunistically at Coos Bay during recruitment season September 9, 2001. H = Haplotype. Sample size = 71.

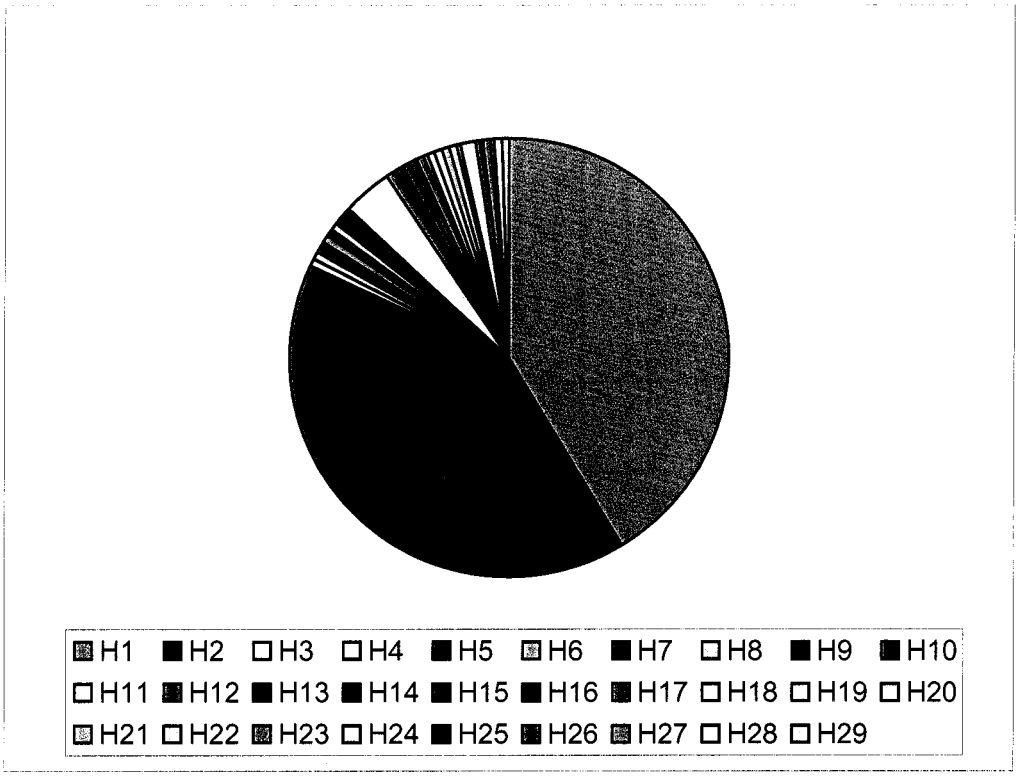

Figure 9. Sequence haplotype frequencies for Dungeness crab megalopae sampled opportunistically at Coos Bay during recruitment season 2001. H = Haplotype. Sample size = 200.

These 29 haplotypes were compared with the haplotypes obtained from 75 individual megalopae collected in the year 1997, 1998, and 1999 from the same site, Coos Bay (Figure 9 and 10). From the pie charts in figure 9 it is clear that the common haplotypes from the year 2001, H1 and H2, are also present in 1997, 1998 and 1999. Compared to 2001 when on some days only one haplotype was present, in 1997, 1998 and 1999 more number of haplotypes are seen and no unique haplotypes are present.

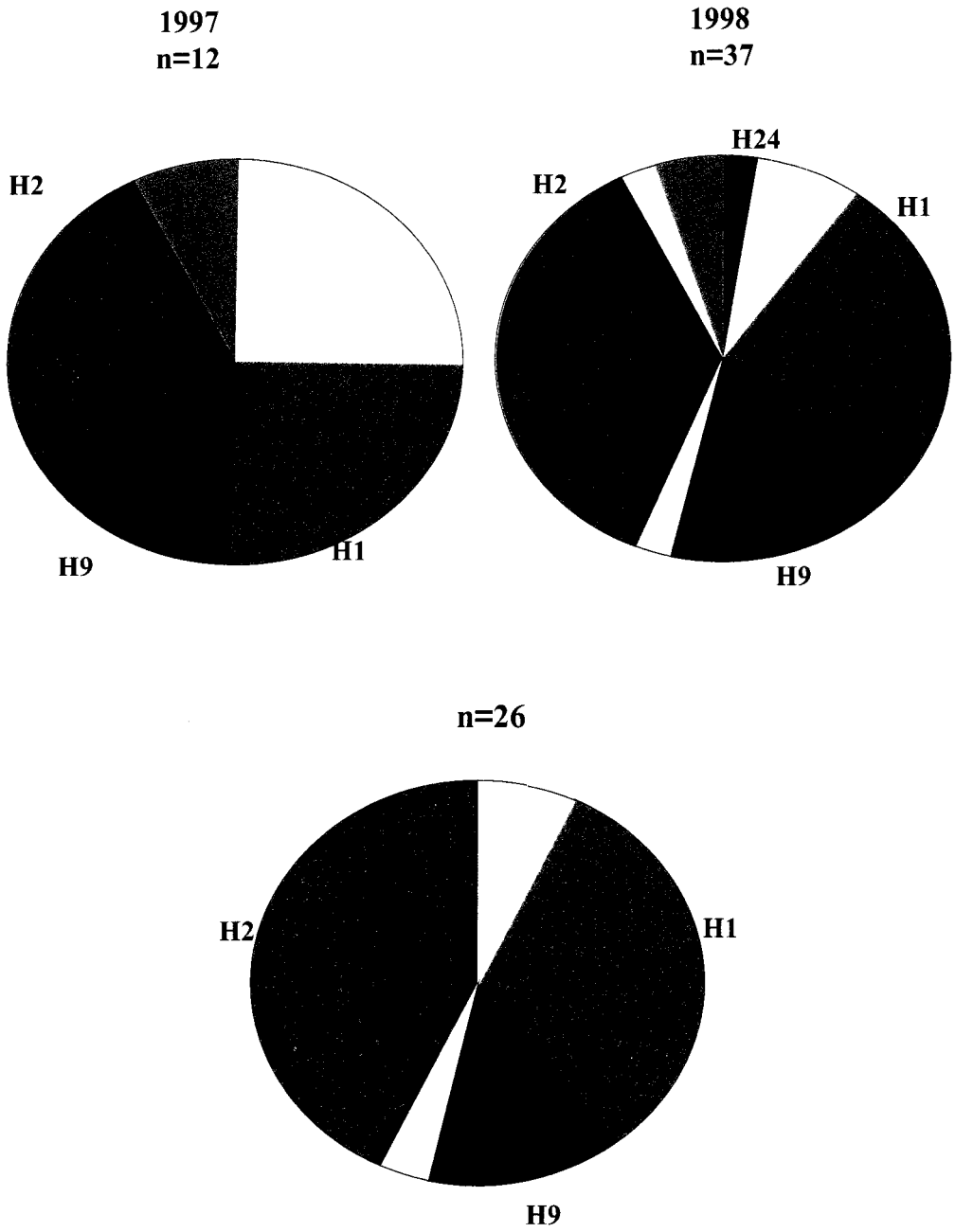

Figure 10. Sequence Haplotype frequencies for Dungeness megalopae sampled opportunistically at Coos bay during recruitment seasons 1997, 1998, and 1999 (Modified from Chokalingam 2003). n = Sample size.

The 275 sequences were aligned and the portion of the COI gene was comprised of 220 positions (sites with gaps/missing data were excluded), of which 41 were variable (Table 7).

Table 7. Sample size and variable sites for different group of populations.

Grouping	Sample size	Number of positions included for alignment	Number of variable sites
2001	200	220	29
1999	26	220	5
1998	50	220	5
1997	12	220	2
All samples	275	220	41

Phylogenetic analysis

Phylogenetic trees were constructed using both a neighbor joining and minimum evolution dendogram and a bootstrap and interior branch test of phylogeny. These trees are not shown here, as all were equivalent. The phylogenetic trees obtained were equivalent regardless of whether minimum evolution and neighbor joining dendogram were constructed using Jukes – Cantor, Tamura-Nei, or Tamura-3-parameter methods. Only the trees constructed (Figures 11 to 13) using Tamura- Nei parameter are shown in all cases.

The phylogenetic trees presented here (Figures 11 to 13) show haplotype designation rather than the name of each sample. Haplotypes H28, H21, and H10 show

more divergence (~ 4 x .005= 0.020) as compared to the most common haplotypes H1 and H2. It can be assumed that the common haplotypes are closer to the ancestral haplotype and the recent haplotypes have diverged more from the common ancestral sequence (Figure 11).

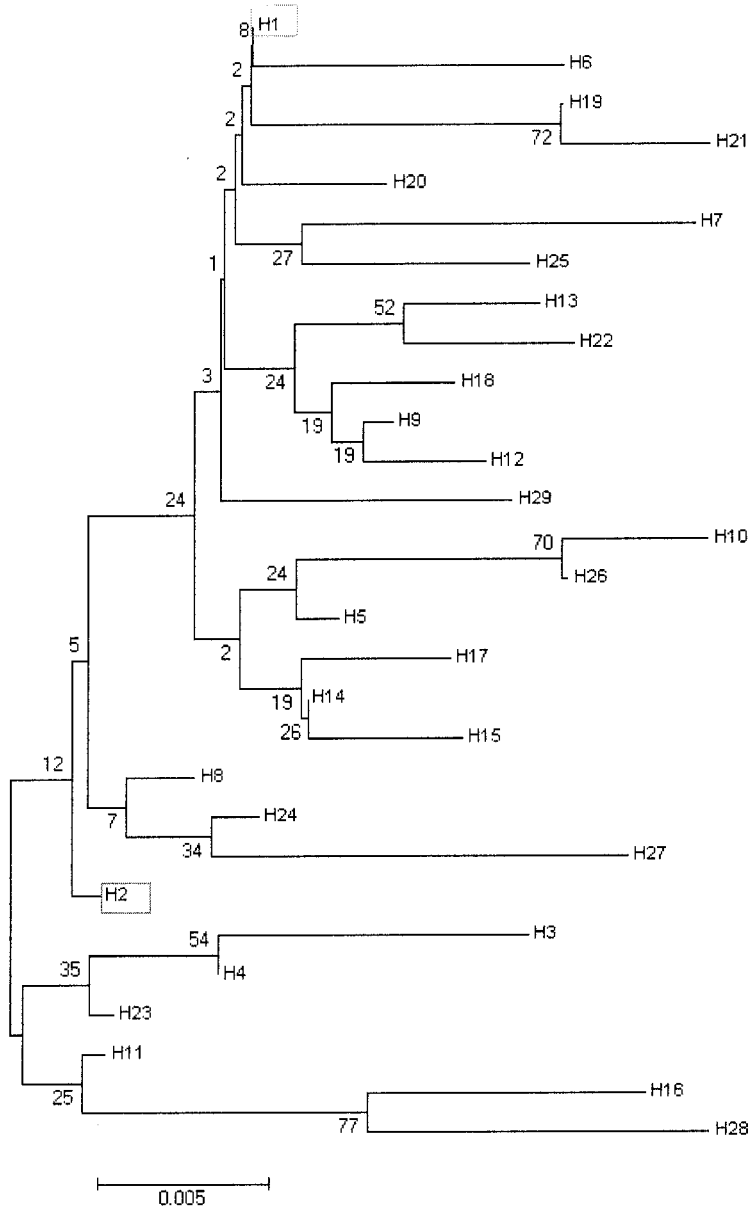

Figure 11. Minimum evolution phylogenetic tree relating to the 29 haplotypes observed among the 200 Dungeness crab mtDNA sequences. The tree represents 500 bootstrap replicates constructed by MEGA. The scale bar represents divergence using the Tamura-nei correction in MEGA.

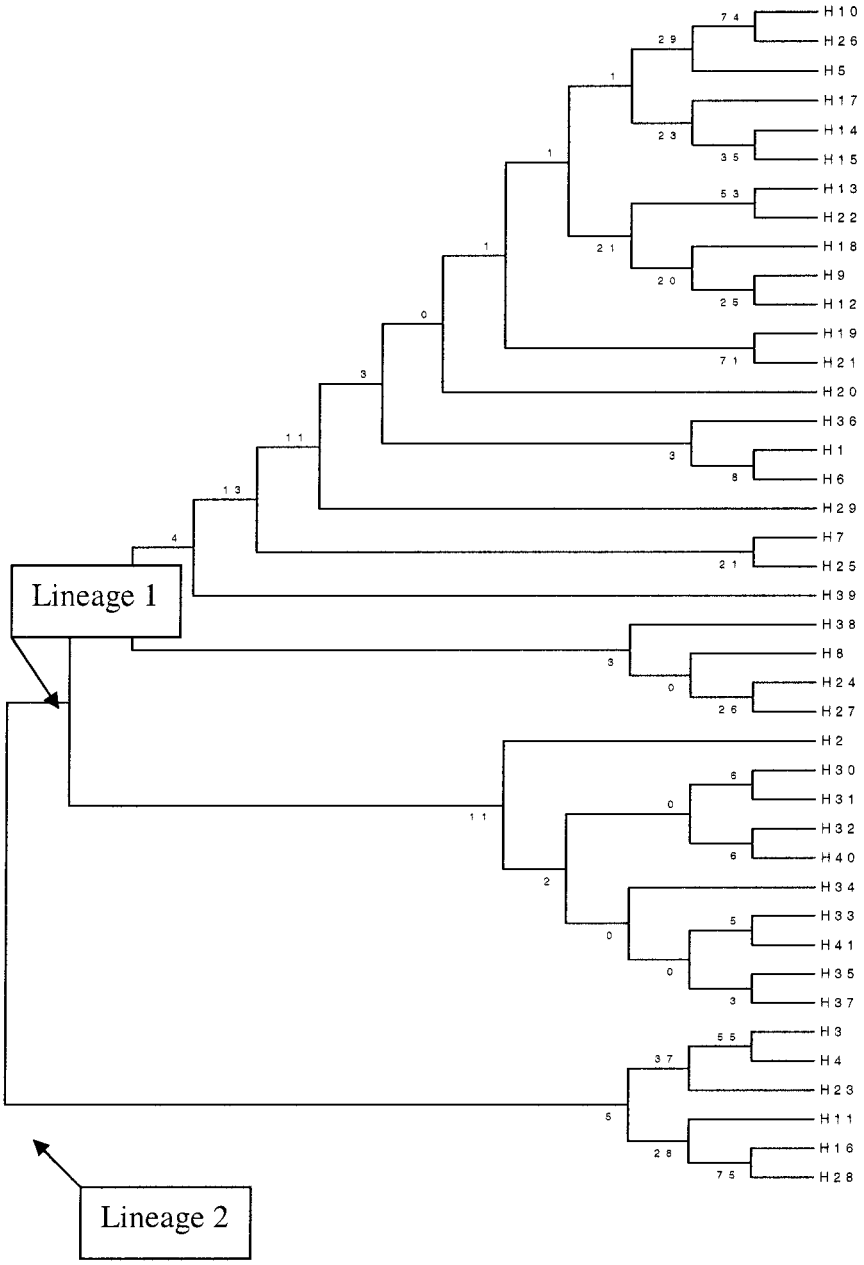

Figure 12. Neighbor joining test of phylogeny relating to the 41 haplotypes observed among the 275 Dungeness crab mtDNA sequences collected during recruitment years 1997, 1998, 1999, and 2001. The tree represents 500 bootstrap replicates constructed by MEGA.

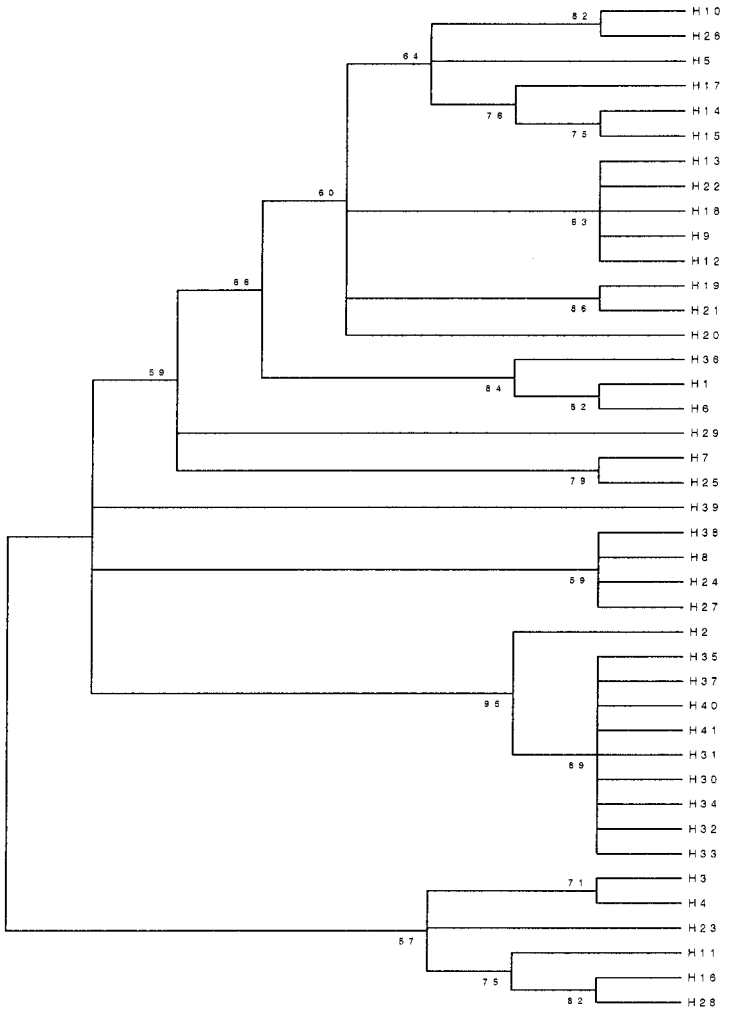

Figure 13. Interior branch test of phylogeny using neighbor joining method relating to the 41 haplotypes observed among the 275 Dungeness crab mtDNA sequences collected during recruitment years 1997, 1998, 1999, and 2001. The tree represents 500 bootstrap replicates constructed by MEGA.

In the phylogenetic analysis the interior branch test of phylogeny and Felsenstein's (1985) bootstrap test was used and then evaluated using Efron's (1982) bootstrap resampling technique. In the case of neighbor joining (NJ) tree (Saitou & Nei

1987), a topology showing the smallest value of the sum of all branches (S) is chosen as an estimate of the correct tree. The S value is not computed for all or many topologies, but the examination of different topologies is imbedded in the algorithm, so that only one final tree is produced. The algorithm of the NJ method is somewhat complicated and is explained in detail in Nei & Kumar (2000). The NJ method produces an unrooted tree, because it does not require the assumption of a constant rate of evolution. Under the minimum evolution criterion the sum, S, of all branch length estimates is computed for all plausible topologies, and the topology that has the smallest S value is chosen as the best tree, the minimum evolution tree. This criterion does not require the evolutionary rate constancy assumption needed in the UPGMA analysis. Figures 11 to 13 represent both neighbor joining and minimum evolution trees using the interior branch test of phylogeny and bootstrap test. Bootstrapping is a computational technique for estimating statistics or parameters when the distribution is difficult to derive analytically (Efron 1982). Felsenstein (1985) introduced its use in phylogeny estimation. Comparisons of an inferred tree with the set of bootstrap replicate trees, typically in the form of tabulation of the proportion of the bootstrap replicates in which each branch from the inferred tree occurs, can give indications of the robustness of the inferred tree. One difficulty with this analysis is the precise interpretation of what these values represent. Usually they are taken as a representation of statistical confidence in the monophyly of groups of sequences, internally related in any manner (Felsenstein 1985). The bootstrap values are very low for minimum evolution as well as neighbor joining trees; among the 41 haplotypes (unique sequences) identified, 90 percent had only one sequence associated

with them. They differ only in 1 to 5 base pairs from each other. It is clear from the phylogenetic trees that these mtDNA haplotypes lack the deep phylogenetic divergences observed in other animals (Avise 1994). This suggests that mtDNA of the COI gene is mutating quickly and these variants are derived by mutation from the common, ancestral sequence.

Discussion

The overall haplotype diversity is high for the samples collected during 2001 (haplotype diversity < 66.50 %). Haplotypes H1 and H2 are present in more than 80% of the individuals. The rest of the haplotypes (H3 to H29) are present in either one or two individuals except H11. All the haplotypes differ little (1 to 5 base pairs) from the common haplotypes. This suggests that these variants are derived from a rapid mutation in the COI gene (Avise 1994). Of the 29 haplotypes identified, 90% of the haplotypes had only one sequence associated with them. This finding is similar to that of Li & Hedgecock (1998), who reported that over 70% of the haplotypes in their study of genetic heterogeneity among larvae of Pacific oysters in Dabob Bay in Washington were singletons (Li and Hedgecock coined the term "singletons" for these haplotypes).

Over 90% of haplotypes observed in this study are singletons, haplotypes found in only a single individual. Such large numbers of rare haplotypes and singletons are unexpected in a small population (Boom et al. 1994). Similar results have been observed in another study done by McMillen-Jackson & Bert (2004) on blue crabs. They investigated the population genetic structure of blue crabs in the waters of the eastern United States using restriction fragment length polymorphism (RFLP) of the mitochondrial DNA molecule. Genetic diversity was notably high; nucleotide diversities were in the range of 1.0 to 2.0%. They observed that 79 % of the haplotypes were singletons. Douglas et al. (2003) did a similar study on Flannelmouth Sucker

(*Catostomus latipinnis*). They found 49 unique haplotypes, 53% of which represented singletons.

Ocean currents play an important role on dispersal and recruitment of megalopae. The Davidson current transports larvae north during the first three months of the year (Johnson et al. 1986). After the spring transition larvae are transported south by the California current. In 2001 the annual current pattern was el Niño, a Southern Oscillation that is characterized by weak upwelling and warmer surface temperatures in the equatorial Pacific Ocean. Thus, the source populations are mostly from the south during the early part of the season 2001.

On two of the days (July 26, 2001, and September 9, 2001), the samples showed only one haplotye (H1). Based on our current data, it can be assumed that the samples are either closely related through common inheritance, or they are from the same or related mother(s). Previous studies have shown that these larvae travel in patches during their dispersal phase. These patches may be composed of larvae released from a single mother or a population of related mothers. On September 9, 2001 the samples showed 29 haplotypes. Roegner & Shanks (2001) suggest these late arriving megalopae are supplied from more northern latitudes during the fall current shift. These northern populations might be more variable. Alternatively, they might be derived from several northern spawning sites. Figures 11 to 13 show two maternal lineages present. This indicates that megalopae arriving at Coos Bay are descended from two maternal lines. All members of the second lineage come from later arriving megalopae. Again, this is evidence that the more northern populations are more

genetically diverse. The tree constructed from the interior branch test of phylogeny is not shown, as it was equivalent. But when we look at the phylogenetic trees constructed by pooling all the samples from four years (Figures 12 and 13) there are three maternal lineages present. The low bootstrap values represent few base pair changes and these values allow rotation around axis.

The interior branch test will show the confidence probability in the Tree Explorer; if this value is greater than 95 percent for a given branch, then the inferred length for that branch is considered significantly positive. It is expected that the branches should bifurcate neatly with a strong support on the branch. However in this case it does not bifurcate but a trifurcation or more is often seen. This can be explained on the basis of the differences in haplotypes, which differ by few base pairs (1 to 5). Note that the topology of the phylogenetic trees is different (Figures 12 and 13). This is expected due to the difference in the statistical programs for the interior branch test and the bootstrap test of phylogeny.

When the haplotypes were compared across all four years it was found that the rare haplotype 24 was present in four individuals in 1998 (Chokalingam 2003) and in only one individual in 2001 (Figures 7 and 9). This could indicate that some of the rare haplotypes are consistently present from year to year. Avise (2000) said that phylogeographical analysis is crucial to understanding processes such as population subdivision, speciation, ecological adaptation, and historical climate change. The results of this study have moved us forward in our understanding of the dynamics of *Cancer magister* population structure and gene flow.

Future Directions

As mentioned, the long-term project objectives of this research are being incorporated into part of a 10-year study to analyze genetic variability amongst various marine wildlife. Ultimately, these data will be used to make recommendations for size and spacing of marine reserves along the Pacific coast. It is essential that the degree of genetic variability present within and between the populations of Dungeness crabs is understood in order to develop effective conservation and management tools.

The main purpose of the current study in particular was to analyze and compare the genetic makeup of recruiting cohorts of *Cancer magister* megalopae at Coos Bay, in the year 2001. The data will be incorporated with other studies to be done in this lab on Dungeness crab populations, which will examine the genetic structure and larval distribution of *Cancer magister* along the Pacific coast.

Further investigation in this lab will include larger sample sizes taken throughout the recruitment season and will include samples of both adult and megalopae along the Pacific coast. These additional studies of both temporal and spatial genetic variation will increase the understanding of the degree of genetic heterogeneity found between populations and will provide an unparalleled measure of larval dispersal and insight into the biogeography of Dungeness crab.

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To whom it may concern,

I am giving permission to Sonali Upadhye to use the behavioral light trap pictures in her M.S. Thesis. I am the original author of this picture.

Sincerely,

Leslee Parr

Leslee Parr, Ph.D.
Assistant Professor Biological Sciences